REMARKS

Sequence Compliance:
The Office Action included a Notice to Comply with Requirements for Patent Applications Containing Nucleotide Sequences and/or Amino Acid Sequence Disclosures. In a telephone conversation with the Examiner, the Examiner acknowledged receipt of a previously filed amendment regarding sequence disclosures correcting the defects in the specification.

Supplemental Material:

Applicants have provided additional material with this Amendment and Response in the form of a 37 CFR 1.132 Declaration. The material consists of experiments performed using Applicants' processes providing delivery of an expressible nucleic acid sequence to which a compound was attached. The experiments show expression of the modified sequence in cell-free extracts, in cells in vitro, and in cells in vivo.

Rejection of claims under 35 U.S.C. 112:

Claims 1-17 have been rejected under §112 as not being enabling for the methods claimed. Specifically, the Office Action states that the examples are not sufficiently representative for the broad range of compounds claimed for attaching to an expressible nucleic acid sequence. The Office Action further states that the description does not distinguish those molecules having the ability to be incorporated within an expressible sequence.

It is the Applicants' opinion that the examples provided are representative of a wide variety of compounds that can be attached to the DNA without disrupting expression. DNP, biotin and digoxin are haptens. In addition, they may also be considered antigens. Biotin is also a ligand which is bound by the proteins Streptavidin and Avidin. Rhodamine and Cy 3 are fluorescent molecules. The NLS is a peptide as well as a highly charged polycation. Because of the charge-charge interaction between anionic nucleic acid and cationic NLS, a person having knowledge in the art would expect that attachment of a polycation to the nucleic acid would inhibit expression. Contrary to this expectation, Applicants found that expression increased in vivo when the nucleic acid was modified by attaching NLS peptides (example 5).

Applicants have amended claims 1 to more distinctly identify the claimed invention. Specifically, claim 1 has been amended to limit the number of modifications allowable on the nucleic acid. Claim 1 further incorporates claim 2: the minimal expression levels for the modified nucleic acid and increased that minimum from "greater than 40%" to "greater than 50%". Support for the amended claim can be found in the examples provided in the specification as well as the supporting document: Slattum et al. Efficient in Vitro and in Vivo Expression of Covalently Modified Plasmid DNA. Molecular Therapy 2003 In press.

The Office Action states that while the specification is enabling for a process for nucleic acid delivery to a cell in vitro, it does not reasonably provide enablement for nucleic acid delivery to a cell in vivo. Applicants respectfully disagree. In this particular example, the location of the cell would not be expected to determine how the cell sees the DNA. The location of the cell should not determine the biochemical characteristics of the enzymes involved in

recognizing and transcribing a given nucleic acid or modified nucleic acid. As evidence of this, Applicants submit the following journal article: Slattam et al. Efficient in Vitro and in Vivo Expression of Covalently Modified Plasmid DNA. Molecular Therapy 2003 In press. Slattum et al. 2003, using the method described in the specification, show that a modified expressible sequence is transcribed and translated in cell-free extracts (page 2-3), in cells in vitro following transfection (page 3), and in liver hepatocytes in vivo following tail vein delivery of the DNA (page 3-4). The same modified DNA could be monitored by fluorescence microscopy (page 4).

In examples 3, 4 and 5 of the Specification, Applicants have demonstrated efficient luciferase expression of the modified nucleic acid in vivo. In some instances, expression was even enhanced relative to unmodified nucleic acid. Applicants also show, in example 6 of the specification (starting on page 26), enhanced immune response against luciferase encoded by a plasmid that was modified within the expressible sequence by attachment of rhodamine prior to delivery of the plasmid to cell in vivo. Applicants believe that example 3-6 of the specification, together with the enclosed journal article, provide strong evidence that the location of the cell is not a determining factor in expression levels of a nucleic acid sequence modified according to the claimed invention.

The Office Action correctly points out that the are several barriers yet to be overcome to fully enable effective gene therapy. Applicants further agree that delivery of a therapeutic gene to a cell in vivo to achieve a therapeutic effect is gene therapy. However, Applicants do not claim that their invention is gene therapy. Applicants also have not claimed delivery of any particular therapeutic gene nor treatment of any particular disease by gene therapy. Rather, Applicants' invention is the delivery of a nucleic acid to a cell wherein the nucleic acid has been chemically modified within an expressible sequence, which is not the equivalent of delivering nucleic acid to the cell for the purposes of gene therapy.

In other words, a nucleic acid that is delivered to a cell for a therapeutic purpose may be modified according to the invention, without the modification inhibiting expression of the therapeutic gene more than 50%. Similarly, an expressible nucleic acid delivered to a cell for research purposes may also be modified within the expressible sequence without inhibiting expression of the sequence more than 50%. The nature of the expressible nucleic acid and purpose for which it is delivered does not affect the claimed process of modifying the nucleic acid within the expressible sequence without inhibiting expression of the sequence more than 50%.

On page 12 of the Office Action, the Examiner points out that Felgner, in WO 99/13719, discusses several DNA labeling procedures that either fail to provide real time detection or disrupt function of the DNA. It is the Applicants' opinion that the belief, as expressed by Feegner in WO 99/13719, that labeling DNA will render the DNA nonfunctional proves the non-obviousness of the applicants' invention. Applicants have clearly shown, in the instant application as well as the enclosed published peer-reviewed journal article, that nucleic acid expressible sequences can be modified such that they are functional. Furthermore, the modifications can be used to track the nucleic acid.

Rejection of claims under 35 U.S.C. 102:

Claims 1-9 and 13 have been rejected as being anticipated by Leahy et al 1996. Applicants have amended the claims to specify a modification level of less than 1 attachment of a

compound per 100 base pairs and an expression level of greater than 50% following the modification. Applicants show in the specification and in the enclosed Slattum et al. document that this level of modification remains detectable and does not inhibit expression of the sequence by more than 50%.

Claim 1, 7 and 13 have been rejected as being anticipated by Ireland et al. 1987. Applicants respectfully disagree. Ireland et al. examined the ability of a cell to repair plasmid DNA damaged by certain DNA alkylating agents and then to stably integrate the repaired DNA into the cell genome. Thus, a successful event, as measured by Ireland et al., required that only one DNA molecule be repaired and integrated in a single cell. It is Applicants' opinion that the analysis of Ireland et al. provides no information on the ability of the modified sequence itself to be expressed.

The Examiner's objections and rejections are now believed to be overcome by this response to the Office Action. In view of Applicants' amendment and arguments, it is submitted that claims 1 and 3-17 should be allowable.

Respectfully submitted,

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doi:10.1016/S1525-0016(03)00152-7

Efficient *in Vitro* and *in Vivo* Expression of Covalently Modified Plasmid DNA

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The tracking of plasmid DNA (pDNA) movement within cells requires the attachment of labels to the DNA in a manner such that: (a) the pDNA remains intact during the labeling process and (b) the labels remain stably attached to the DNA. Keeping these two criteria in mind, we have recently developed a series of alkylating reagents that facilitate the one-step, covalent attachment of compounds directly onto nucleic acids in a nondestructive manner. Using these DNA-alkylating reagents, we have attached a wide range of both fluorescent and nonfluorescent reporter molecules onto pDNAs. We now show that even with the covalent attachment of various marker compounds, the pDNA remains expression competent. The ability to create labeled, expression-competent DNA allows for the simultaneous tracking of both pDNA location and reporter gene expression within living or fixed cells.

Key Words: transfection, DNA labeling, gene expression, covalent modification, DNA tracking

Introduction

The transfection of plasmid DNA into the nucleus of a cell is a complicated and poorly understood process. Even when using the best reagents currently available, transfection success can vary widely from one cell line to the next and a significant number of cell lines remain refractory to transfection. The biological basis for such cell-to-cell variations in transfection capabilities is being intensively studied.

One approach for developing a better understanding of the transfection process is to visualize the transfected DNA directly within cells in culture. Along these lines, many current studies use fluorescently labeled DNA to follow or "track" the DNA as it enters and moves around inside of a cell. However, to gain truly relevant information in these types of studies, it is important that the labeled DNA being tracked is intact plasmid DNA and not just small, labeled DNA fragments. The importance of this is underscored by the fact that it is well known that small DNAs (i.e., oligonucleotides or small DNA fragments) can move around within cells much less hindered than large DNAs and consequently the intracellular distribution pattern following transfection for fluorescently labeled "small" nucleic acids is very different from that for large DNAs [1]. A second and equally important factor is that the fluorescent label be attached to the plasmid DNA through a linkage that does not allow the label to be easily cleaved from the nucleic acid. For these reasons, we have recently developed a series of labeling reagents that facilitate the covalent attachment of various fluorescent and nonfluorescent compounds directly onto the nucleic acid via a convenient, one-step reaction that leaves the plasmid intact. These reagents (*Label*IT), whose reactive center is an aromatic nitrogen mustard, covalently alkylate DNA, primarily at N^7 of guanine residues [2]. Labeling with these reagents allows for the fluorescent tracking of intact plasmid DNA molecules within individual cells following transfection, electroporation, or microinjection.

We now show that even after the covalent attachment of any of a variety of compounds (fluorophores, haptens, hydrophobic compounds) to plasmid DNA (pDNA), efficient expression of the labeled pDNA can be maintained. This was unexpected given that it is generally believed that covalent attachment would inhibit transcription and therefore expression [3,15]. In addition to being versatile, this also creates an advantageous situation in which pDNAs that are both expression competent and fluorescently labeled can be visually monitored while being transported to the nucleus so that DNA localization can be correlated with expression.

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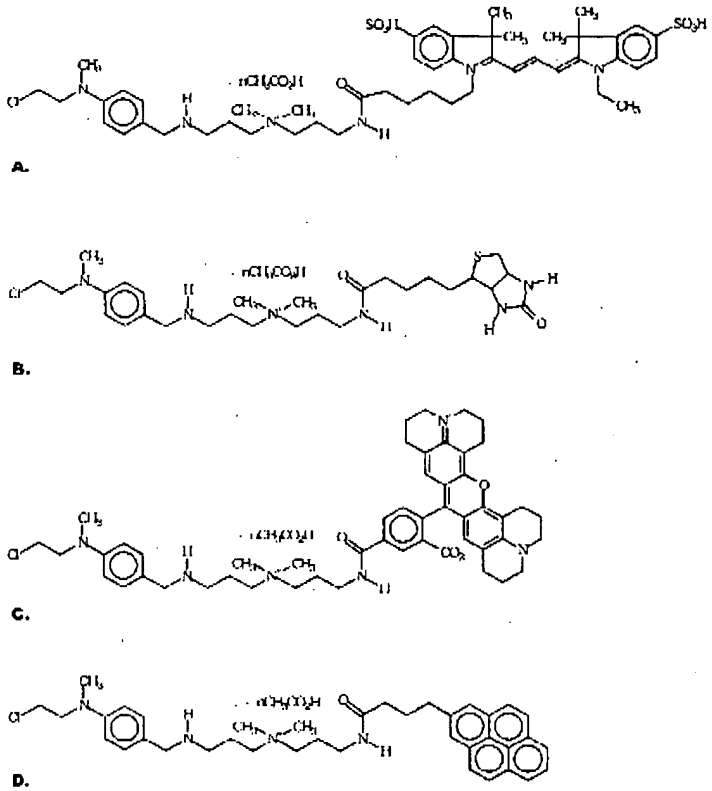

FIG. 1. Chemical structures of the DNA labeling reagents. (A) Structure of *Label*IT-CY3. (B) Structure of *Label*IT-biotin. (C) Structure of *Label*IT-CX rhodamine. (D) Structure of *Label*IT-pyrene.

RESULTS

Covalent Attachment of Labels to DNA

We labeled pDNA using the alkylating reagents depicted in Fig. 1 (i.e., *Label*IT reagents). The number of covalently attached labels on the pDNA was determined by spectrofluorometric analysis (data not shown). Covalent attachment was visualized using gel electrophoresis in which total fluorescence, gel-shift retardation, and activity of attached markers were monitored (Fig. 2). Following labeling with *Label*IT CX-rhodamine, fluorescent DNA is visible in the absence of ethidium bromide staining due to the covalently attached rhodamine (Fig. 2, Unstained, top, CX-rhodamine lanes). When we used increasing amounts of labeling reagent to label the DNA, increased fluorescent signal was observed (compare CX-rhodamine lanes .2 and .1). When we used hydrolyzed reagent (*Label*IT CX-rhodamine) as a control, covalent labeling was not observed (i.e., DNA could be seen only with ethidium bromide staining; see CX-rhodamine lane H). The hydrolyzed reagents represent appropriate controls for this study because although they can interact with DNA via electrostatic binding (similar to unhydrolyzed, active reagents), they are unable to react covalently with the nucleic acid. DNA labeled with Cy3 is not visible without ethidium bromide staining, although the labeled DNA (Fig. 2, Cy3 lanes .1 and .2) exhibits a detectable gel retardation compared to the hydrolyzed control and the unlabeled plasmid control (lanes Cy3 H and C). The Cy3-labeled DNA is not visible in the unstained picture because the excitation wavelength of the gel box is 302 nm and Cy3 has essentially no absorption at this wavelength and therefore cannot fluoresce. The gel retardation observed for the labeled DNA is partially due to decreased total negative charge of the plasmid DNA when the fluorophore-containing reagent (with cationic linker) is attached. Covalent attachment of biotin to DNA is demonstrated by both gel retardation (biotin lanes) and a supershift (with smearing) that is detectable after the addition of streptavidin to the samples (Fig. 2, biotin lanes .1 and .2 with and without streptavidin). Addition of streptavidin had no effect on DNA that had no biotins attached (see biotin + streptavidin, lane H).

Expression of Labeled Plasmids Using *in Vitro* Transcription/Translation Reactions

To determine whether plasmid DNA retained gene expression competency following covalent labeling, we per-

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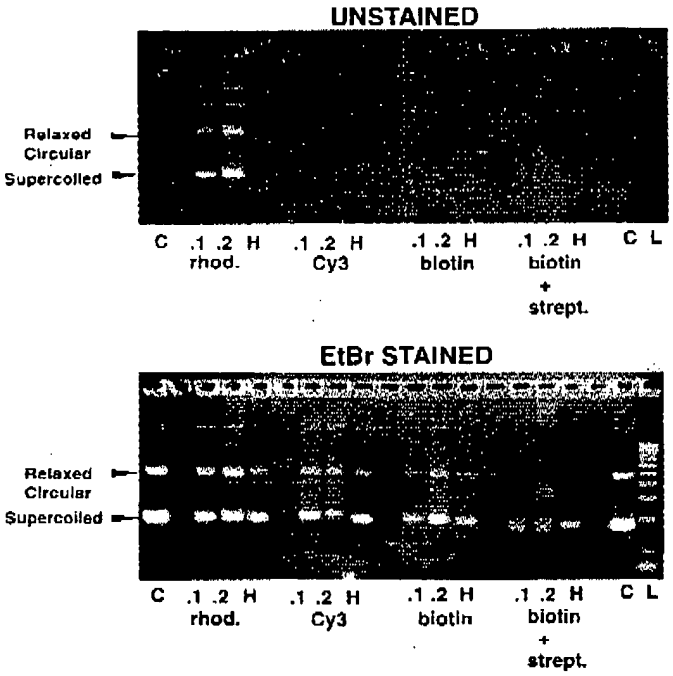

FIG. 2. Agarose gel electrophoresis of labeled DNA in which the same gel is displayed before staining (Unstained) and after ethidium bromide staining (EtBr Stained). Lanes C contain unlabeled pCI Luc plasmid. CX-rhodamine lanes .1 and .2 contain DNA labeled at a 0.1:1 and a 0.2:1 (wt/wt) ratio, respectively; CX-rhodamine lane H contains DNA reacted at a 0.2:1 (wt/wt) ratio with the hydrolyzed reagent. Cy3 lanes .1 and .2 contain DNA labeled at a 0.1:1 and a 0.2:1 (wt/wt) ratio, respectively; Cy3 lane H contains DNA reacted at a 0.2:1 (wt/wt) ratio with the hydrolyzed reagent. Biotin lanes .1 and .2 contain DNA labeled at a 0.1:1 and a 0.2:1 (wt/wt) ratio, respectively; biotin lane H contains DNA reacted at a 0.2:1 (wt/wt) ratio with the hydrolyzed reagent. Biotin + streptavidin lanes .1, .2, and H contain the same DNA as the biotin wells, but were mixed with 1 μg streptavidin prior to gel analysis. Lane L contains a 1 kb DNA ladder.

formed the following experiment. We labeled plasmid DNA (pCILuc) encoding luciferase (under the control of a CMV enhancer/promoter and/or T7 promoter) at several different labeling densities using each of four different labels (CX-rhodamine, Cy3, pyrene, and biotin) and tested for expression using a coupled *in vitro* transcription/translation kit (TnT Kit; Promega Corp., Madison, WI). All expression data that were generated using the labeled plasmids were normalized to expression results obtained using an unlabeled control plasmid. The results shown in Fig. 3 demonstrate that the efficiency of expression between labeled plasmids and unlabeled control plasmid is nearly equivalent when the number of labels per plasmid is between 5 and 10. However, as the numbers of attached labels per plasmid increase, the resulting expression levels drop off rapidly although there is some dependence on the type of label attached. When labeling density reaches 45–55 labels per plasmid, luciferase expression is less than 10% of unlabeled pDNA regardless of the label type.

Expression of Labeled Plasmids in Transfected Cells

We also evaluated the ability of labeled DNA to express in cells in culture relative to unmodified plasmid DNA. We transfected labeled DNA (pCILuc) into Hepa 1-CLC6 and COS 7 cells using *Trans*IT LT-1 (Mirus Corp.) and harvested the cells 48 h after transfection and luciferase activity was determined (Fig. 4). All expression data obtained for labeled plasmids were normalized to expression results obtained using an unlabeled control plasmid. In Hepa cells, the labeled DNA retained expression capabilities of approximately 75% of the control plasmid at modification levels of 5–10 labels per plasmid (Fig. 4A). However, in contrast to the *in vitro* TnT reactions (transcription/translation), when there were 25–30 labels per plasmid, substantial transgene expression was still maintained (~50% unmodified DNA levels). Also, considerable expression (~25%) was retained even at higher modification levels (~50 labels per plasmid).

High levels of transgene expression (luciferase) were also maintained when we transfected labeled DNA into COS 7 cells, although overall the reductions in expression from the labeling were slightly lower (Fig. 4B). In addition, the effect of labeling was more dependent on the type of label (Fig. 4B). Nonetheless, the pyrene-labeled DNA expressed more efficiently at every level of modification. As with the Hepa cell transfections, the most striking result is the considerably higher level of expression from more heavily modified plasmids compared to the cell-free transcription/translation results.

In Vivo Expression of Labeled Plasmids

Last, we tested labeled plasmids for expression capabilities *in vivo* (in ICR mice; Harlan Laboratories). Liver delivery was accomplished by high-volume, tail vein injection us-

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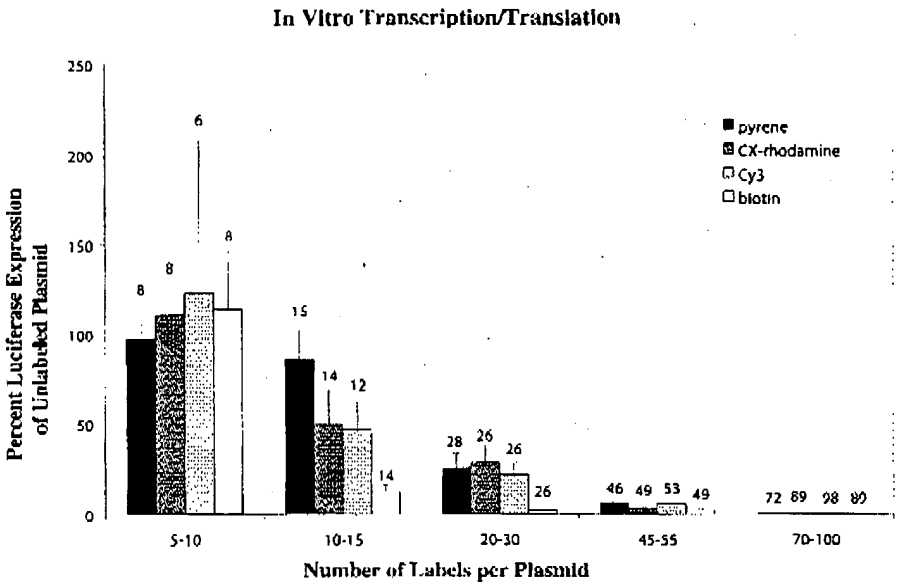

FIG. 3. *In vitro* transcription/translation efficiency of a series of labeled plasmids is shown. Results are shown as ratios of luciferase expression of the labeled plasmid divided by the unlabeled plasmid control. (Data shown are from 2 experimental days; on one of the days duplicate reactions were performed and on the other day single reactions were performed. Error bars were obtained using the coefficient of variation.) Results are grouped by the number of labels per plasmid and by the label type (CX-rhodamine, Cy3, pyrene, and biotin). The actual number of labels per plasmid is shown at the top of each error bar. The numbers of labels per plasmid for biotin are included as estimated values only.

ing 10 μg of labeled plasmid DNA (in Ringers buffer) per mouse [4,5]. We sacrificed the mice at 24 h postinjection and harvested the livers, homogenized them in cell lysis F5 buffer, and assayed them for luciferase activity (Fig. 5). The control mice injected with the unmodified plasmid produced an average of 3.34 μg of luciferase per liver. All expression data obtained following injections of labeled plasmids were normalized to expression results obtained for the unlabeled plasmid injections. Mean luciferase production for mice injected with DNA modified at a level of approximately 10 labels per plasmid showed little or no loss in activity compared to the unlabeled plasmid injections. When we increased the level of modification to 20 labels per plasmid the mean luciferase production decreased, but statistically retained expression levels indistinguishable from the unmodified control plasmid (mean expression values showed no difference using the Student *t* test).

Fluorescence Tracking (and Expression) Using Labeled DNA

To demonstrate the utility of the technique for tracking labeled pDNA in cultured cells, we covalently modified an expression vector encoding enhanced yellow fluorescent protein (pEYFP-Nuc) with rhodamine and transfected it into COS 7 cells. At various time points after transfection we detected red (rhodamine) and yellow (EYFP) fluores- F6 cence using confocal microscopy (Fig. 6). In this experiment we observed that by 1 h posttransfection, the majority of the cells had fluorescent spots on the plasma membrane or inside the cell. By 4 h posttransfection nearly 100% of the cells had internalized the fluorescently labeled DNA (small punctate dots) and low-level EYFP expression was observable in a low percentage of cells. By 24 h posttransfection, high-level EYFP expression was observed in 90–100% of the cells. Interestingly, by 24 h the distribution of rhodamine-labeled DNA had changed dramatically in most cells from a punctate (dot) to a much more diffuse (cloud) pattern. This is likely a result of the labeled plasmid DNA becoming released from the endosomal compartments.

To determine if fluorescently labeled pDNA and reporter gene expression could be observed within the same cells *in vivo*, we labeled an expression plasmid encoding enhanced green fluorescent protein (pEGFP) with Cy3 and injected it into the tail vein of ICR mice. We sacrificed the mice at ~24 h postinjection, froze the livers, and prepared thin sections (7 μm). In all liver hepatocytes expressing EGFP, fluorescently labeled DNA (Cy3 signal) was also present in a punctate staining pattern. The vast majority of the Cy3-fluorescent signal was located in the cytoplasm, although some dots were observed in nuclei (Figs. 7A7D). In a significant number of cells, we observed F7 a small amount of Cy3-labeled DNA (~1–5 dots/cell) in the cytoplasm although no EGFP expression was detected. However, in general, the cells that contained the largest number of dots were usually the ones displaying the highest levels of EGFP expression. In a very small number of cells (<0.1%) we found large numbers of fluorescent dots in the cells but detected no EGFP expression (data not shown).

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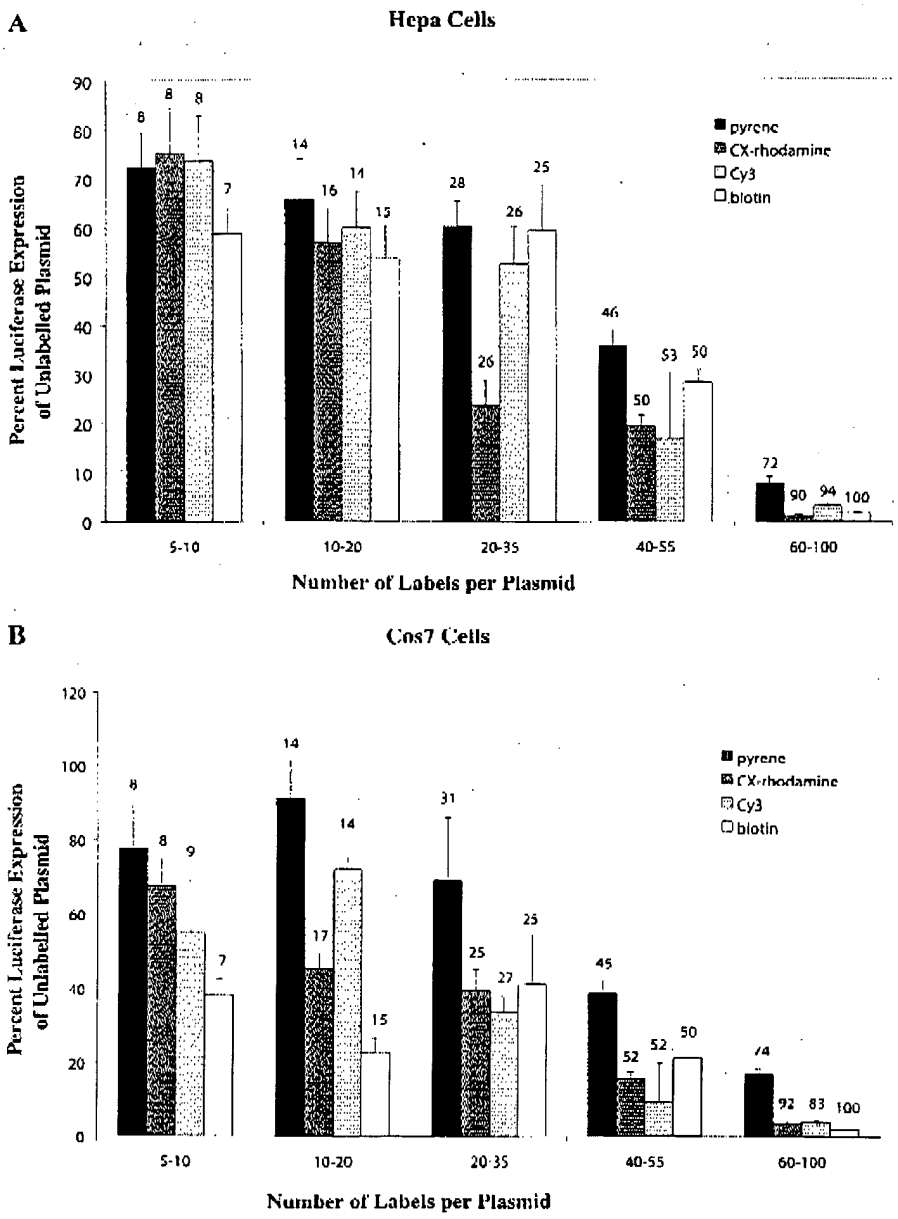

FIG. 4. Expression efficiency of labeled pCILuc plasmid in (A) Hepa cells or (B) COS 7 cells. Results are shown as ratios of luciferase expression of the labeled plasmid divided by the unlabeled plasmid control. Total luciferase production per well for the unlabeled control was 3.0 and 8.7 μg of luciferase per well in Hepa and COS 7 cell transfections, respectively. Results are grouped by the number of labels per plasmid and by the label type (CX-rhodamine, Cy3, pyrene, and biotin). The actual number of labels per plasmid is shown at the top of each error bar. The numbers of labels per plasmid for biotin are included as estimated values only. For Hepa cells duplicate experiments were performed on 3 days (n = 4–6) (error bars are obtained from the average coefficient of variation). In COS 7 cells duplicate experiments were performed on 2 days (n = 2–4) (error bars are obtained from the average coefficient of variation).

Discussion

The key to finding new ways to increase nonviral gene delivery capabilities in living cells ultimately lies in our ability to understand better the barriers in the transfection process. In this regard, new and sophisticated DNA labeling tools are needed to assist in the delineation of these intracellular barriers. Currently, a variety of methods are available for attaching fluorescent compounds onto DNA but none of these methods allow for the direct covalent attachment of fluorescent compounds directly onto genes (or plasmid DNA) while at the same time leaving the gene intact. For this purpose, a new class of

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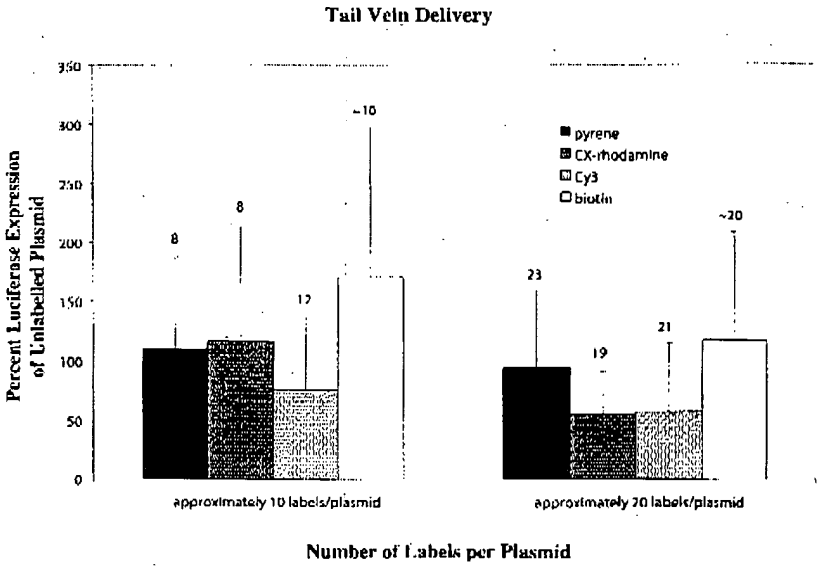

FIG. 5. High-pressure tail vein injection transfection efficiency into mouse livers is shown for a series of injections using labeled pCI Luc (CMV promoter, luciferase transgene). Results ($n = 5$) are shown as ratios of luciferase expression of the labeled plasmid to that of the unlabeled plasmid control. Total liver luciferase production for the unlabeled control was 2.4 μg. Results are grouped by number of labels per plasmid and by the label type (CX-rhodamine, Cy3, pyrene, and biotin). The actual number of labels per plasmid is shown on top of each error bar. The numbers of labels per plasmid for biotin are included as estimated values only. Error bars were obtained by taking the square root of the sum of the squares of the standard deviations of labeled and unlabeled plasmids.

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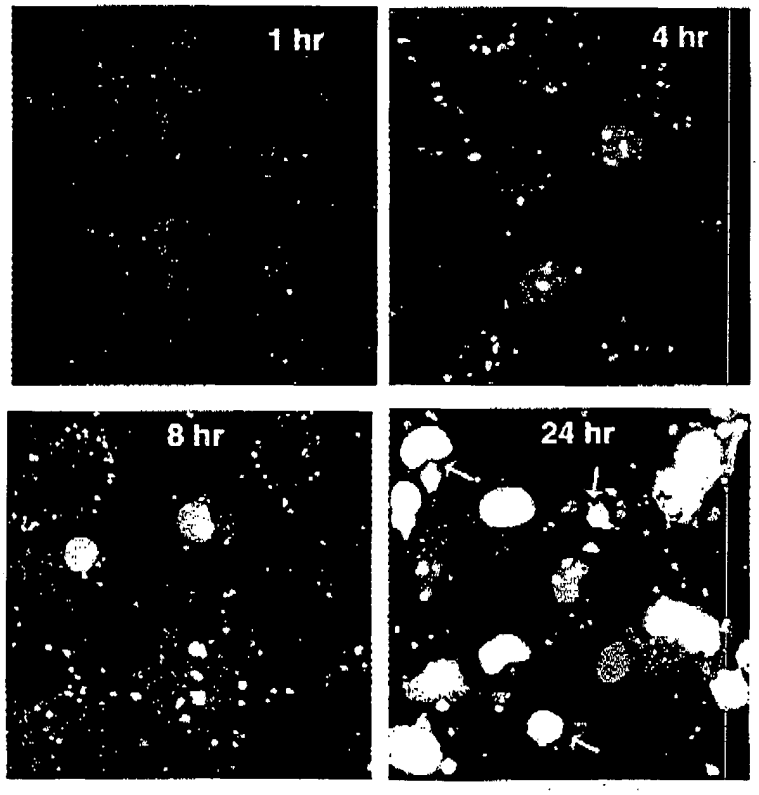

FIG. 6. Confocal fluorescence microscopy of COS 7 cells transfected (with *TransIT* LT-1) with 2 μg EYFP-Nuc plasmid DNA labeled at a 0.1:1 (wt/wt) ratio for 1 h at 37°C (approximately 25 labels/plasmid). Photomicrographs taken 48 h after transfection. Images represent COS 7 cells 1 h after transfection with rhodamine-labeled pDNA, 4 h posttransfection, 8 h posttransfection, and 24 h posttransfection. Note the changes in labeled pDNA (red) localization from the surface of the cells to a perinuclear location during the time progression (small arrows in the 24-h image). The overall increase in rhodamine fluorescence at the 24-h time point is likely a result of escape of labeled DNA from the endosomal compartment.

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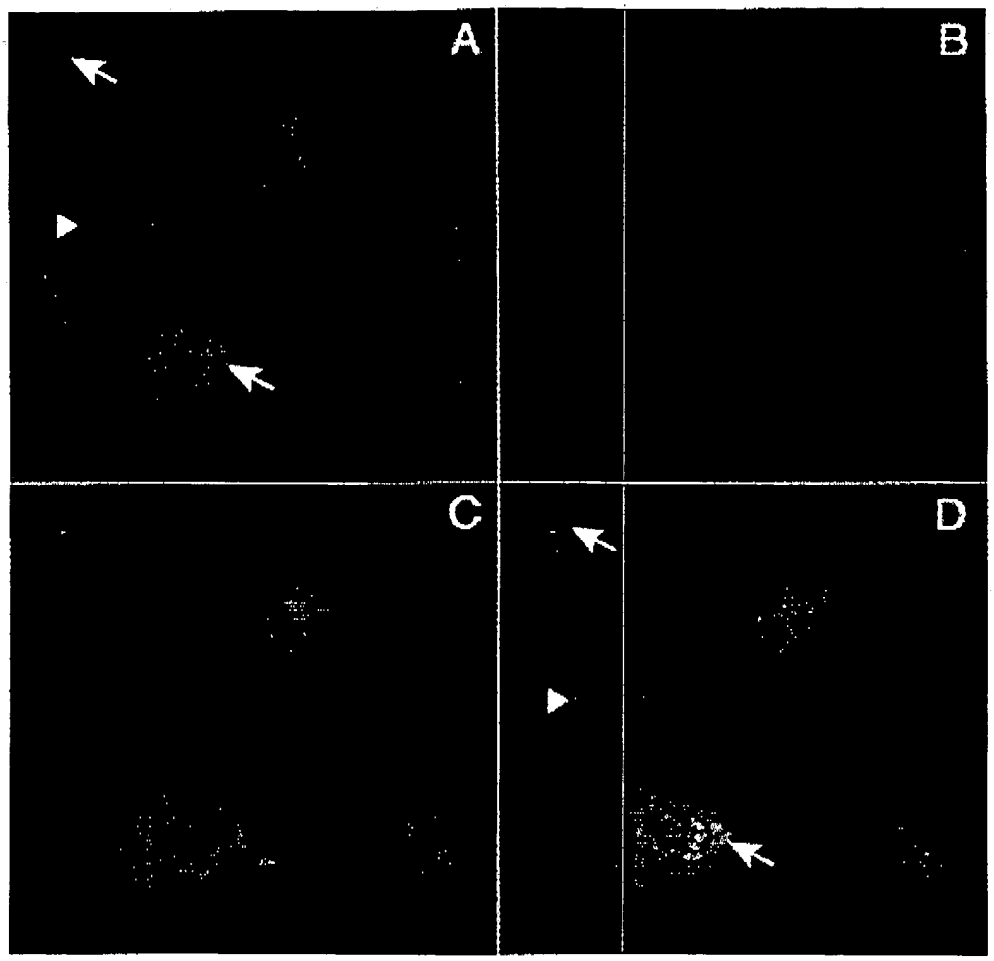

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FIG. 7. Confocal fluorescence microscopy of mouse liver hepatocytes following tail vein delivery of 10 μg EGFP plasmid DNA labeled with LabelIT Cy3 (at a 0.1:1 (wt/wt) ratio for 1 h at 37°C—approximately 25 labels/plasmid). Photograph taken 24 h after tail vein injection. (A) Cy3-labeled DNA (red). (B) Nuclei of liver cells (blue DAPI staining). (C) Green indicates green fluorescent protein expression (EGFP). (D) An overlay of all three channels. Arrows in A and D indicate Cy3-labeled DNA within GFP-expressing hepatocytes. Arrowheads indicate Cy3-labeled DNA in cells in which EGFP is not detectable.

chemical labeling reagents (*Label*IT reagents) that facilitate the covalent attachment of fluorophores (and nonfluorescent labels) directly to the nucleic acid bases in a nondestructive manner was developed. These reagents covalently modify nucleic acids via an alkylation reaction and they have been shown to work effectively on DNA of any size or conformation (P.S., M.-A.W., K.M., J.H., unpublished results). In addition, they function well in the attachment of a variety of different labels (fluorescent or nonfluorescent, hydrophilic or hydrophobic).

In this study we now demonstrate that even after covalent attachment of multiple labels directly onto the plasmid DNA, the encoded transgene remains expression competent. At lower levels of labeling (5–10 labels per plasmid) expression of the transgene was either unaffected (*in vitro* transcription/translation reactions and *in vivo* experiments) or minimally reduced (cell culture transfection experiments). As expected, the overall level of gene expression competency was inversely proportional to the number of attached labels. But surprisingly, even when the DNA was labeled at a level such that labels were attached at sites within the expression cassette (i.e., promoter, coding sequence, and 3′ UTR), efficient gene expression still occurred (the expression cassette consists of approximately 3 kb of the 5.8-kb pCILuc plasmid). We have confirmed that the expression cassettes of ~100% of

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the plasmids are modified (using *Label*IT-biotin) when using a labeling ratio of ≥0.025:1 (wt/wt; *Label*IT-biotin/pDNA) by gel retardation experiments with streptavidin (data not shown). At the labeling ratio of 0.025:1 (wt/wt, *Label*IT/pDNA) between 6 and 10 labels are attached to the pCILuc plasmid. We have also observed that at these lower levels of DNA labeling, restriction digestion of the labeled DNA remains essentially unaffected (data not shown).

While labeled DNA could be efficiently expressed both *in vitro* and *in vivo*, there were differences among the levels of gene expression inhibition observed in the three types of expression systems (transcription/translation-coupled reactions, *in vitro* transfections, *in vivo* gene delivery). Most remarkable was the effect observed in mouse liver cells *in vivo* (via intravascular delivery of naked DNA), where modified plasmid DNA with approximately 10 labels per plasmid (~1 label/500 bp) maintained expression capabilities very similar to those of unmodified plasmid DNA. Even when labeling levels of ~20 labels per plasmid were used, transgene expression *in vivo* was ≥50% unmodified plasmid DNA levels for all four types of labels tested. The variation in expression among the three experimental conditions (transcription/translation reaction, cultured cells, and liver *in vivo*) could be due to different rate-limiting steps within these systems. One specific factor that could account for some of the differences between the *in vitro* and the *in vivo* results is the effect of the nucleotide repair system within cells *in vivo* (see below).

It is important to note that even though a small reduction in expression levels was observed when using the labeled DNA, the absolute levels of gene expression following the gene transfer step in each of these experiments remain quite high. Thus, the level of expression inhibition observed following covalent attachment of the fluorophore does not have a dramatic effect on the overall percentage of cells expressing the transgene. This is visually demonstrated in Fig. 6, for which rhodamine-labeled DNA (~25 labels per plasmid) was transfected into COS 7 cells and the cells were monitored for uptake of fluorescently labeled DNA and expression of yellow fluorescent protein simultaneously. As can be seen, nearly 100% of the cells display both types of fluorescence, indicating that the labeled DNA has been internalized by each of the cells and expression of the transgene has taken place.

Possibly the most important aspect of these findings lies in its therapeutic potential. The fact that expression capabilities are maintained following the attachment of labels with very different properties (hydrophobic, hydrophilic, charge) means that this labeling technology could be easily used to attach various functional compounds to expression cassette-containing plasmid DNAs for the purpose of increasing gene transfer and/or gene expression. This potential is especially relevant in light of the recent progress made in increasing the potency of nonviral gene transfer vectors, in particular naked DNA delivery *in vivo* [6–14]. Thus, even modest enhancements in gene expression gained via the attachment of novel ligands or endosomal lytic or transport molecules could have profound effects on the utility of nonviral vectors for human gene therapy interventions.

A variety of mechanisms may explain how gene expression competency is maintained following labeling. The two most likely scenarios include transcriptional readthrough of the modified bases or repair of modified bases. In two previous reports, it was found that up to 40% of a plasmid's transgene expression capabilities were maintained following transfection into NIH3T3 cells of pDNA covalently modified using photoactivatible chemical labeling reagents [15,16]. These authors attributed the gene expression capabilities to the ability of eukaryotic RNA polymerase II to read through the modified bases. Alternatively, repair of modified bases could be taking place, thereby allowing efficient expression of the modified genes. This could be carried out through either a nucleotide excision repair or a base excision repair mechanism. There may also be a transcription-linked repair mechanism whereby only the labeled bases inside the coding cassette get repaired. In this study, the level of gene expression observed following covalent labeling with the alkylating agents was significantly higher than that observed in these previous studies (i.e., photolabeling). We are currently actively investigating the precise mechanism by which these cells enable transgene expression of covalently modified genes.

In summary, these labeling reagents represent highly useful tools for studying both DNA transport and its resulting reporter gene expression. By simply adjusting the ratio of labeling reagent to DNA during the labeling reaction, it is possible to: (a) control the absolute level of DNA labeling and (b) identify plasmid DNA labeling levels in which the DNA can be easily visualized while still allowing for high-level gene expression *in vitro* or *in vivo*. Furthermore, the ability of covalently modified DNA to be expressed suggests that this gene chemistry approach could be used to attach ligands or other chemical moieties to facilitate increased cellular uptake or better transport within the cell (e.g., nuclear transport signals for nuclear localization) [17].

Materials and Methods

Plasmid DNA. For all labeling and expression studies involving luciferase, the 5865-bp pDNA pCILuc in the closed, circular form was used [18]. The luciferase expression cassette contained within this plasmid is approximately 3030 bp. This includes the entire expression cassette, poly(A) tail, and CMV enhancer/promoter. For fluorescence/expression studies, the plasmids pEYFP-Nuc (Clontech) and pEGFP (Clontech) were used. Plasmid EYFP-Nuc encodes an enhanced yellow fluorescent protein that localizes to the nucleus and pEGFP encodes a green fluorescent protein that remains predominantly in the cytoplasm.

DNA labeling. The nucleic acid labeling reagents *Label*IT-biotin, *Label*IT-CX-rhodamine, *Label*IT-pyrene, and *Label*IT-Cy3 were obtained from

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Mirus Corp. Synthesis of the *LabelIT* reagents is described in U.S. Patent US06262252. Chemical structures of the reagents are shown in Fig. 1. DNA (pCI Luc) was modified using *LabelIT* reagents according to the manufacturer's recommendations; briefly, *LabelIT* reagent and DNA were combined in 20 mM Mops buffer, pH 7.5 (100 μg DNA/ml buffer) at various wt/wt ratios (reagent/DNA) and incubated at 37°C for 1 h. The labeled pDNA was separated from unattached label by ethanol precipitation, using 1/10 volume 5 M NaCl as the counterion. The purified pDNA was reconstituted in 20 mM Mops buffer, pH 7.5.

Hydrolysis of labeling reagents (alkylating group). The nitrogen mustard responsible for nucleic acid labeling in the *LabelIT* reagents was hydrolyzed (inactivated) by incubating in 20 mM Mops buffer, pH 7.5, for 1 h at 80°C. The hydrolysis (replacement of chlorine moiety by a hydroxyl group) was confirmed by mass spectrometry (data not shown). The hydrolyzed reagents were used as a control in labeling reactions to demonstrate that the *LabelIT* reagents are covalently linked to the DNA and not merely electrostatically associated with the DNA (Fig. 2).

Determination of labeling density. The number of groups attached to DNA was estimated using published molar extinction coefficients (ε), expressed as M^{-1} cm^{-1}, of the various marker groups: CX-rhodamine, 80,000; Cy3, 150,000; and pyrene, 43,000 (Molecular Probes and Amersham) In some cases λ_{max} was observed to shift after the attachment of the fluorophore to the DNA. Labeled DNA was scanned on a UV spectrometer (Beckman DU 530) to obtain the actual λ_{max} experimentally. The λ_{max} for CX-rhodamine, Cy3, and pyrene were determined to be 586, 548, and 350 nm, respectively. DNA molar concentration was determined by measuring the absorbance at 260 nm and dividing by 660 g DNA/mol DNA bp. The number of biotin molecules cannot be determined by measuring absorption. Biotin Incorporation was assumed to parallel CX-rhodamine and pyrene incorporation because all three labeling reagents carry two positive charges under the conditions of the labeling reaction. The incorporation of *LabelIT* reagents is approximately linear with respect to weight ratio. A reaction under the above conditions using a weight ratio of 0.2:1 (*LabelIT* reagent:DNA) would result in a labeled pCILuc plasmid containing approximately 50 labels.

In vitro transcription/translation. All *in vitro* transcription/translation (TnT) experiments were done using the commercially available TnT Quick Coupled Transcription/Translation Kit (Promega). TnT experiments were carried out according to the manufacturer's protocol.

Cell transfections. Transfections were performed on COS 7 (large T antigen-transformed monkey kidney cells) and Hepa 1-CLC6 (mouse hepatobiliary carcinoma) and were carried out using Mirus's *TransIT* LT-1 transfection reagent. For each transfection, 2 μg of plasmid DNA (labeled or unlabeled) was complexed with LT-1 and then added to growing cells according to the manufacturer's recommendations. For reporter gene assays, transfected cells were harvested after ~48 h, and cell lysates were prepared and assayed for luciferase activity. For fluorescence microscopy studies, cells were grown on glass coverslips in 35-mm wells and transfected, and then coverslips were washed (phosphate-buffered saline), fixed with glutaraldehyde, and mounted onto glass slides.

Tail vein injections. Mouse tail vein injections were performed as previously described [4]. Briefly, 10 μg of plasmid DNA encoding the reporter gene luciferase (in 2 ml of Ringer's solution) was injected rapidly into the tail vein of 20- to 25-g ICR mice (Harlan Laboratories, Indianapolis, IN). Twenty-four hours postinjection the mice were sacrificed and the livers were removed. For luciferase detection, liver tissue was homogenized in cell lysis buffer (100 mM KH_2PO_4, pH 7.8, 1 mM DTT, 0.1% Triton X-100) and assayed for luciferase activity as previously described [10]. For *in situ* expression studies using labeled plasmid DNA and fluorescent reporter proteins (pEGFP), livers were removed, quick frozen in liquid nitrogen in tissue mounting medium (OCT, Tissue Tek), and thin sectioned on a cryostat (Zeiss Instruments). The resulting 7-μm sections were mounted onto glass slides (with coverslips) and analyzed via confocal microscopy.

Fluorescence microscopy. All fluorescence microscopy was performed using an LSM 510 (Zeiss) scanning confocal microscope.

Acknowledgments

This work was supported in part by NIH SBIR Grant 5-R44-GM58297. LabelIT and TransIT are registered trademarks of Mirus Corp. Cy3 is a registered trademark of Amersham Biosciences.

RECEIVED FOR PUBLICATION NOVEMBER 20, 2002; ACCEPTED APRIL 24, 2003.

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